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Cerebellar Hypoplasia in the Gunn Rat is Associated with Quantitative Changes in Neurotypic and Gliotypic Proteins

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We are characterizing toxicant-induced injury to the nervous system by measuring nervous system, cell-type specific proteins together with accompanying changes in morphology and behavior. In the present study, cerebellar neurotoxicity was assessed in the Gunn rat, an autosomal recessive mutant that exhibits degeneration of Purkinje cells due to hereditary hyperbilirubinemia. Five proteins associated with neuronal or glial cell types were chosen for evaluation as follows: 1) G-substrate, a Purkinje cell-specific phosphoprotein that serves as the endogenous substrate of cyclic GMP-dependent protein kinase; 2) PCPP-260, a Purkinje cell-specific phosphoprotein that serves as an endogenous substrate of cyclic AMP-dependent protein kinase; 3) synapsin I, a synapse-specific phosphoprotein present in all neurons; 4) glial fibrillary acidic protein, an astrocyte-specific protein; and 5) myelin basic protein, a protein unique to myelin. In comparison to heterozygote (J/) controls, homozygous (//) rats showed alterations in the amounts of neurotypic and gliotypic proteins in cerebellum that were consistent with the neuropathological effects associated with development of hyperbilirubinemia in the Gunn rat. Decreased cerebellar cyclic GMP, but not cyclic AMP, alterations in indices of motoric competence and increased responsiveness to a nociceptive stimulus also were observed in // rats. In general, the degree of cerebellar hypoplasia was predictive of the degree of biochemical, morphological or behavioral change observed. The results indicate that neurotypic and gliotyic proteins may be used as biochemical indicators of neurotoxicity.

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Cerebellar Hypoplasia in the Gunn Rat is Associated with Quantitative Changes in Neurotypic and Gliotypic Proteins[1]

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ABSTRACT

We are characterizing toxicant-induced injury to the nervous system by measuring nervous system, cell-type specific proteins together with accompanying changes in morphology and behavior. In the present study, cerebellar neurotoxicity was assessed in the Gunn rat, an autosomal recessive mutant that exhibits degeneration of Purkinje cells due to hereditary hyperbilirubinemia. Five proteins associated with neuronal or glial cell types were chosen for evaluation as follows: 1) G-substrate, a Purkinje cell-specific phosphoprotein that serves as the endogenous substrate of cyclic GMP-dependent protein kinase; 2) PCPP-260, a Purkinje cell-specific phosphoprotein that serves as an endogenous substrate of cyclic AMP-dependent protein kinase; 3) synapsin I, a synapse-specific phosphoprotein present in all neurons; 4) glial fibrillary acidic protein, an astrocyte-specific protein; and 5) myelin basic protein, a protein unique to myelin. In comparison to heterozygote (*Jj*) controls, homozygous (*jj*) rats showed alterations in the amounts of neurotypic and gliotypic proteins in cerebellum that were consistent with the neuropathological effects associated with development of hyperbilirubinemia in the Gunn rat. Decreased cerebellar cyclic GMP, but not cyclic AMP, alterations in indices of motoric competence and increased responsiveness to a nociceptive stimulus also were observed in jj rats. In general, the degree of cerebellar hypoplasia was predictive of the degree of biochemical, morphological or behavioral change observed. The results indicate that neurotypic and gliotyic proteins may be used as biochemical indicators of neurotoxicity.

Exposure to neurotoxicants often results in cytopathologic changes that are concentrated in specific regions of the CNS and that preferentially involve specific types of neurons or glia (Herndon and Coyle, 1978; Spencer and Schaumberg, 1980). Because the cellular heterogeneity of the developing and mature mammalian CNS is expressed by unique neurotypic and gliotypic proteins, we proposed that these nervous system-specific proteins may be used as biochemical indicators of neurotoxicity (O'Callaghan and Miller, 1983). We are testing this hypothesis by administering known neurotoxicants to the developing and mature rat and assessing the effects of these agents on previously characterized nervous system-specific proteins (see O'Callaghan *et al.*, 1983b; O'Callaghan and Miller, 1984; Miller and O'Callaghan, 1984) together with an examination of the accompanying changes in morphology (O'Callaghan and Miller, 1984; Miller and O'Callaghan, 1984) and behavior (Miller *et al.*, 1982; Walsh *et al.*, 1982; Miller, 1984; Miller and O'Callaghan, 1984).

The cerebellum of the immature and adult rat often has been shown to be susceptible to toxic insult (Herndon and Coyle, 1978; Reuhl and Chang, 1979; Phillips and Cragg, 1982; Fishman and Yanai, 1983). One extensively studied model for toxicant-induced damage to the cerebellum is the Gunn rat, an autosomal recessive mutant that exhibits life-long hyperbilirubinemia due to a lack of UDP-glucuronyl transferase (Blanc and Johnson, 1959). The elevated serum bilirubin found shortly after birth in homozygous recessive Gunn rats is associated with the development of bilirubin encephalopathy (kernicterus), the hallmark of which is marked cerebellar hypoplasia (Sawasaki *et al.*, 1976; Tamaki and Yamasaki, 1976; Tamaki *et al.*, 1977). This condition is ameliorated by phototherapy to degrade serum bilirubin (Johnson and Schutta, 1970; Sisson *et al.*, 1974) or, conversely, is exacerbated by agents that displace bilirubin from serum albumin (Schutta and Johnson, 1969; Rose and Wisniewski, 1979), suggesting that bilirubin is responsible for the observed pathology. Histological (Schutta and Johnson, 1967; Sawasaki *et al.*, 1976) and immunohistochemi-

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ABBREVIATIONS: CNS, central nervous system; cGMP, cyclic GMP; cAMP, cyclic AMP; PCPP-260, Purkinje cell-specific phosphoprotein (M_r 260K); GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; RIA, radioimmunoassay; IgG, immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RSA, relative specific activity; CNPase, 2′-3′-cyclic nucleotide 3′-phosphohydrolase.

cal (Mikoshiba *et al.*, 1980, 1982) examinations of the Gunn rat cerebellum indicate that Purkinje cells are affected preferentially with the greatest damage occurring in lobules that mature postnatally. A reduction in the number of granule cells and hypertrophy of astrocytes also have been observed (Mikoshiba *et al.*, 1980, 1982).

In the present study we used the Gunn rat as a model for evaluating nervous system-specific proteins as biochemical indicators of cerebellar neurotoxicity. By measuring neurotypic and gliotypic proteins associated with cerebellar cell types, we expected to find changes consistent with the cytopathology associated with bilirubin encephalopathy. Five such proteins that have been characterized on the basis of biochemical and physiochemical properties and that have been localized by immunocytochemistry were chosen for evaluation: 1) G-substrate, the endogenous substrate for cGMP-dependent protein kinase (Schlichter *et al.*, 1978, 1980) that is specific to the soluble compartment of cerebellar Purkinje cells (Schlichter *et al.*, 1980; Detre *et al.*, 1984; Nestler and Greengard, 1984); 2) PCPP-260, a membrane-bound Purkinje cell-specific phosphoprotein (M_r260K) that serves as an endogenous substrate for cAMP-dependent protein kinase (Walaas *et al.*, 1985, 1986) and is apparently identical to the previously described p400 protein (Mallet *et al.*, 1976; Mikoshiba *et al.*, 1979); 3) synapsin I, a synapse-specific phosphoprotein associated with synaptic vesicles, apparently in all synapses (De Camilli *et al.*, 1983a,b; Huttner *et al.*, 1983; Nestler and Greengard, 1983); 4) GFAP, an astrocyte-specific protein (Bignami and Dahl, 1977; Eng, 1980) and; 5) MBP, a protein unique to myelin (Sternberger *et al.*, 1978; Norton, 1981; Hartman *et al.*, 1982). Other measurements included cyclic nucleotide concentration in specific cerebellar lobes, light microscopy and behavioral assessments of motoric competence and sensory function. The data obtained were consistent with the neurotoxic effects associated with hyperbilirubinemia in the Gunn rat.

Materials and Methods

Materials. Bolton-Hunter reagent, [γ-^{32}P]ATP, [^{125}I]Protein A, [^{3}H]cGMP, [^{3}H]cAMP and cGMP and cAMP RIA kits were purchased from New England Nuclear (Boston, MA). G-Substrate and corresponding antisera were the gifts of Dr. Angus C. Nairn (Rockefeller University). Synapsin I and corresponding antisera were the gifts of Drs. Jesse Chan and Eva Perdahl (Rockefeller University). Antiserum to GFAP was obtained from Dako Corporation (Santa Barbara, CA). Monoclonal anti-MBP was obtained from Hybritech (San Diego, CA) and affinity-purified rabbit antimouse IgG was purchased from Pel-Freez (Rogers, AK). Nonidet P-40 was from Particle Data Labs (Elmhurst, IL). Pansorbin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, cAMP, Tris and dithiothreitol were from Calbiochem (San Diego, CA). Bovine serum albumin, Pepstatin A, protein kinase catalytic subunit, sucrose, Amido black 10B and molecular weight standards were from Sigma Chemical Company (St. Louis, MO). Nitrocellulose paper (0.20 μM pore size) was purchased from Schleicher and Schuell (Keene, NH). Other materials used in the RIAs were from the sources described (Goelz *et al.*, 1981; Nairn *et al.*, 1982; Jahn *et al.*, 1984). All reagents used for SDS-PAGE with the exception of Tris (Calbiochem) were obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of analytical reagent grade and were purchased from various commercial sources.

Subjects. Male, homozygous (*jj*) and heterozygous (*Jj*) Gunn rats were purchased from Blue Spruce Farms (Altamont, NY). This strain was originally obtained from the National Institutes of Health (Bethesda, MD) and is characterized by an agouti hood that is brown in appearance. The animals weighed 300 to 500 g and were housed two per cage in a temperature (22°C ± 2)- and humidity (50% ± 10)-controlled colony room supplied with filtered air under positive pressure ventilation. Food (Purina Rat Chow) and water were available *ad libitum*. Fluorescent lighting was maintained on a 12-hr light:12-hr dark cycle beginning at 6:00 A.M. Subjects were divided into four groups. Group 1 (10 months old) was used for histology; group 2 (10 months old) for determination of G-substrate, synapsin I, GFAP and MBP; group 3 (2–4 months old) for the determination of cGMP and cAMP; and group 4 (4 months old) for determination of motoric competence and PCPP-260. Animals in groups 1, 2 and 4 were killed by cervical dislocation; animals in group 3 were killed by focused microwave irradiation (see below).

Histology. Immediately after sacrifice a blood sample was obtained from the aorta for the determination of serum bilirubin and the animals then were perfused transcardially with 0.9% saline followed by 10% Formalin buffered to pH 7.4. The brains were removed, weighed and sliced in saggital section at midline. Beginning at midline, sequential saggital sections 10-μ thick were cut from the right side of each brain. Sections were stained with cresyl violet.

RIA of Neurotypic and Gliotypic Proteins

Tissue preparation. After sacrifice and collection of blood samples, brains were excised from the skull and weighed. The cerebella were dissected, weighed and homogenized in 20 volumes of 1% SDS heated to 90–95°C. Samples were stored at −70°C before RIA.

RIA of G-substrate. G-substrate was measured by nonequilibrium RIA as described by Nairn *et al.* (1982). Pure G-substrate was iodinated with Bolton-Hunter reagent and recovered from the reaction mixture as described (Goelz *et al.*, 1981). Before assay cerebellar samples were diluted 1:30 in a sample buffer containing 150 mM NaCl, 15 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.02% sodium azide and 1% Nonidet P-40. Each sample was assayed in quadruplicate using the microtiter plate procedure as described (Nairn *et al.*, 1982). In this and all other RIAs for neurotypic and gliotypic proteins, a single *Jj* sample was used to construct a standard curve for the determination of the relative amount of a given protein in samples obtained from *Jj* and *jj* subjects. By comparing the immunoreactivity of a specific protein in the sample of interest with the immunoreactivity in the sample used to construct the standard curve, the amount of this protein relative to that in the standard was obtained. This expression of immunoreactivity hereafter is referred to as RSA. The RSA of a particular protein in a given sample was multiplied by a constant that resulted in a mean RSA of 1.0 for each control group. The data are presented on both a total and a concentration basis, *i.e.*, RSA per total tissue and RSA per milligram of tissue protein.

RIA of synapsin I. Synapsin I was measured by detergent-based RIA according to the procedure of Goelz *et al.* (1981). Pure synapsin I was iodinated with Bolton-Hunter reagent and recovered from the reaction mixture as described (Goelz *et al.*, 1981). Before assay samples were diluted 1:50 in RIA buffer (Goelz *et al.*, 1981). Samples were assayed in quadruplicate using the microtiter plate system as described previously (Nairn *et al.*, 1982).

RIA of GFAP. GFAP was assayed by a modification of the dot-immunobinding procedure of Jahn *et al.*, (1984). Gelatin (0.5%) was used in the blocking solution. A single *Jj* sample, diluted in sample buffer (Jahn *et al.*, 1984), was used to prepare the standard curve; all other samples were diluted with sample buffer to a concentration of 0.1 mg/ml and then applied to nitrocellulose sheets in two 5-μl portions per spot (1.0 μg of protein). Anti-GFAP antibody was used at a dilution of 1:500. A linear relationship was observed between the amount of homogenate assayed and the amount of bound [^{125}I]protein A (fig. 1). The specificity of the dot-immunobinding procedure was determined by assaying a sample of *Jj* cerebellum that had been subjected to SDS-PAGE and then electroblotted (Burnette, 1981) onto sheets of nitrocellulose. Radioactivity was bound only to a band corresponding to the electrophoretic mobility of GFAP (fig. 1). In addition, analysis of immunoblots of samples resolved by 2-dimensional electrophoresis (O'Farrell, 1975) revealed that antisera to GFAP bound to a protein

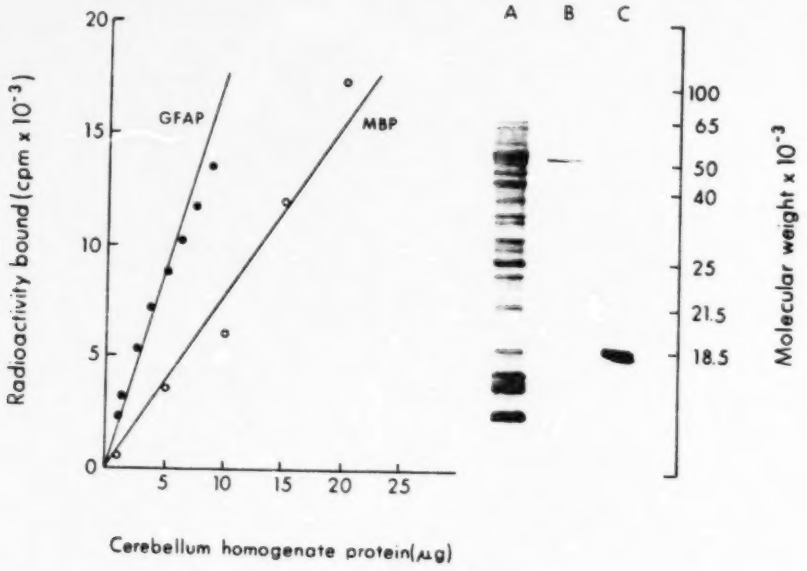

Fig. 1. Relationship between the amount of *Jj* cerebellar homogenate protein and the amount of GFAP and MBP as determined by dot-immunobinding assay (left) and specificity of antisera as determined from western blots of total cerebellar protein (right). Antibody stocks were diluted 1:500. A, electrophoretogram of *Jj* cerebellar homogenate proteins blotted onto nitrocellulose and stained with Amido black 10B. B, corresponding autoradiograph obtained after immunobinding with anti-GFAP. C, corresponding autoradiograph obtained after immunobinding with anti-MBP.

with both the isoelectric and molecular weight characteristics of G... (data not shown).

RIA of MBP. MBP was assayed by a modification of the dot-immunobinding procedure of Jahn *et al.*, (1984). Gelatin (0.5%) was used in the blocking solution. Samples were diluted in sample buffer to a concentration of 1.0 mg/ml and applied to nitrocellulose sheets in two 5-μl portions per spot (10 μg of total protein). Incubation of the nitrocellulose sheets with monoclonal anti-MBP (1:500) was followed by washing in Tris-buffered saline and additional incubation in blocking solution for 30 min (see Jahn *et al.*, 1984). This step was followed by incubation in rabbit antimouse IgG (1:200) for 2 hr with subsequent washing and blocking before incubation in [^{125}I]protein A. The linearity and specificity of the assay were confirmed as described above for GFAP. Because the anti-MBP used reacts with large MBP from rat (Sires *et al.*, 1981), only the prelarge (21.5K) and large (18.5K) forms of MBP (see Colman *et al.*, 1982) showed binding of radioactivity (fig. 1). Myelin isolated from rat (Norton and Poduslo, 1973) showed identical specificity (data not shown).

Detection and Quantification of PCPP-260

Tissue preparation. After sacrifice, blood samples were obtained and cerebella were dissected and weighed as described above. Cerebella were homogenized with a glass-Teflon homogenizer (20 strokes in a Kontes No. 20 vessel) and a total particulate fraction was prepared as described (Walaas *et al.*, 1985, 1986).

Endogenous phosphorylation. To detect PCPP-260 in particulate fractions from cerebellum, tissue samples were assayed for endogenous protein kinase activity in the absence or presence of cAMP, essentially as described (Walaas *et al.*, 1985, 1986). The standard assay contained (final volume, 200 μl): 100 μg of particulate fraction protein, 25 mM Tris (pH 7.4), 6 mM MgSO4, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N′-tetracetic acid, 1 mM dithiothreitol and 5 μM ATP containing [^{32}P]ATP (1×10^{-7} dpm/nmol) in the absence or presence of 10 μM cAMP. Assays were conducted at 30°C after preincubation for 60 sec at 30°C. The reaction was initiated by the addition of a 25-μl aliquot of the particulate fraction and was terminated 60 sec later by the addition of an SDS-PAGE sample buffer (O'Callaghan *et al.*, 1983b). Samples were heated to 90–95°C and the phosphoproteins were resolved by SDS-PAGE as described previously (O'Callaghan *et al.*, 1983b). The acrylamide concentration was 4 and 8% in the stacking and resolving gels, respectively. Gels were stained, destained, dried and processed for autoradiography as described previously (O'Callaghan and Miller, 1984). Molecular weight standards were used to estimate the relative molecular mass of the resolved proteins.

Phosphorylation of acid extracts. PCPP-260 was quantified by "back-phosphorylation" of acid extracts of the total particulate fraction from cerebellum. The details of this procedure have been described by Walaas and co-workers (1985, 1986). Briefly, a total particulate fraction was prepared from cerebellum, without washing. This fraction was resuspended in standard buffer at a concentration of 5 mg of protein per ml. Five milliliters of 5 mM zinc acetate were added to a 0.1-ml aliquot of the resuspended fraction. The precipitated proteins were recovered by centrifugation and then extracted with 10 mM citrate-phosphate buffer (pH 2.8) containing 0.1% Triton X-100 and 2 μg/ml of Pepstatin A. After centrifugation the supernatants were recovered, adjusted to pH 6.0 with 0.5 M Na_2HPO_4 and the final extract was prepared by recentrifugation. The protein content in the final extracts was adjusted so that all samples were of equal protein concentration before phosphorylation. Equal aliquots of the acid extracts then were subjected to back-phosphorylation by the addition of catalytic subunit of cAMP-dependent protein kinase as described (Walaas *et al.*, 1985, 1986). The phosphorylated proteins were resolved by SDS-PAGE and the ^{32}P-labeled bands were detected by autoradiography of the dried gels as described above. The incorporation of ^{32}P into PCPP-260 was quantified by microdensitometry of the autoradiographs using an LKB Ultrascan Laser Densitometer (LKB Instruments, Gaithersburg, MD) as described (O'Callaghan *et al.*, 1983b). For the purpose of consistency (see section on RIA of neurotypic and gliotypic proteins), the arbitrary units of density corresponding to individual PCPP-260 bands were referred to as RSA. The RSA of PCPP-260 in a given sample was then multiplied by a constant that resulted in a mean RSA of 1.0 for each control group. The data are presented on both a total and a concentration basis as described above under RIA of neurotypic and gliotypic proteins.

RIA of cGMP and cAMP

Tissue preparation and dissection. Rats were killed by exposure to focused microwave irradiation generated by a Gerling Moore, Metabostat (model 4095, 3.5 kW for 2.8–3.0 sec). This duration of exposure raised intrahypothalamic temperature to 95°C (O'Callaghan *et al.*, 1983a) and abolished phosphodiesterase activity (data not shown). The brain was removed as described (O'Callaghan *et al.* 1983a) and cut in

saggital section at midline. The cerebellum was then removed from one brain half and cerebellar lobules V, VI and IX (Pellegrino *et al.*, 1979) were dissected from a 1-mm thick saggital section sliced from the medial face of the other cerebellar hemisphere (see O'Callaghan *et al.*, 1983a).

RIA of cGMP and cAMP. Hemicerebella were homogenized in 100 volumes of 0.4 N $HClO_4$ using a glass/Teflon homogenizer. Dissected cerebellar lobules were homogenized by sonification in 100 volumes of 0.4 N $HClO_4$. After centrifugation at 22,000 × *g* for 15 min the supernatants were recovered and frozen (−70°C) for subsequent assay. Pellets were dissolved in 1 N NaOH for protein analysis. Before assay, the thawed supernatants were adjusted to pH 6.0 with 2 M Tris-HCl. The amount of cGMP and cAMP in the neutralized samples was determined in triplicate by RIA (Steiner *et al.*, 1972) after acetylation of samples and standards according to the procedure of Harper and Brooker (1975). Recovery of [^{3}H]cGMP or [^{3}H]cAMP added to the homogenates was found to be 99 ± 1.5%.

Assays for Bilirubin and Protein

Determination of serum bilirubin. Total serum bilirubin was determined using the diazotized sulfanilic acid method of Ertingshausen *et al.* (1973).

Protein assays. Protein was determined according to the method of Lowry *et al.* (1951) with the exception of the experiments concerning the detection and measurement of PCPP-260, where the method of Bradford (1976) was used. Bovine serum albumin was used as a standard in the former, whereas bovine γ-globulin was used in the latter.

Neurobehavioral Assessments

Locomotor activity. Spontaneous locomotor activity was measured in figure-eight mazes (Reiter *et al.*, 1975). This automated monitoring device consists of a series of interconnected alleys (10 × 10 cm) converging on a central arena and is covered with transparent acrylic plastic. Activity, *i.e.*, photocell interruptions, was detected by eight phototransistor/photodiode pairs. Rats were tested for a single 1-hr period during the diurnal portion of the light/dark cycle and photodiode interruptions were recorded every 10 min.

Coordination and balance. A 4-cm wide and 90-cm long wood plank inclined 20° from horizontal and elevated 90 cm off the floor was used to test for balance and coordination. A rat was placed on the elevated end of the plank and allowed to traverse the 90-cm distance to a level platform. Each rat was given three trials and a trial was terminated when 1) the rat fell off, 2) 120 sec had passed or 3) the rat reached the platform at the end of the plank. Latency to reach the level platform was recorded.

Nociception. Sensitivity to a nociceptive stimulus was assessed by the hot-plate procedure (O'Callaghan and Holtzman, 1975); the surface temperature was maintained at 52°C. Licking of the fore or hind paws was taken as the endpoint for the determination of response latencies. Each animal was subjected to two trials. The first served to acquaint the subject with the test environment; response latency in the second trial was the recorded value.

Statistical analyses. The statistical analysis system (SAS, 1982) was used for data analyses. Group mean comparisons were made by the *t* statistic for independent groups or by repeated measure analysis of variance (Keppel, 1973). When group variances were unequal, comparisons were made by T, an approximate *t* statistic with Satterthwaite's approximation for the degrees of freedom. In certain instances associations between measures were assessed by Pearson product moment correlations. Additional details concerning the statistical evaluation for a particular measure appear in the legends to individual figures or tables.

Results

Histology. In agreement with previous observations (Schutta and Johnson, 1967; Tamaki and Yamasaki, 1976), we found that in comparison to *Jj* controls (fig. 2A) *jj* rats with confirmed hyperbilirubinemia exhibited cerebellar hypoplasia that varied from moderate (fig. 2B) to severe (fig. 2C). Cerebellar lobules from moderately affected *jj* subjects were comparable to those of *Jj* controls; the arrangement and appearance of Purkinje cells and the thickness of the molecular and granular layers also were unremarkable at the light microscopic level. In contrast, severely affected *jj* subjects showed a marked derangement of cerebellar lobules that mature postnatally (Altman, 1969) as well as a loss of Purkinje cells and shrinkage of molecular and granular layers in the remaining lobules. Purkinje cells in the intact lobules were less numerous, smaller and often pyknotic (fig. 2C′).

RIA of neurotypic and gliotypic proteins. Body and brain weights were reduced significantly in the jaundiced (*jj*) subjects (table 1). Although cerebellar weights were reduced by 50%, this decrease did not account entirely for the reduction in brain weight because values for brain weight minus cerebellum also were depressed (table 1). In *jj* rats values for total G-substrate, synapsin I and MBP were reduced significantly. These decreases were not a reflection of the reduced cerebellar weights in *jj* rats because values for concentration of G-substrate and synapsin I also were decreased (64 and 38%, respectively) whereas those for MBP were slightly increased (23%). These findings are indicative of a marked loss of Purkinje cells, a reduction in granule cells or other cerebellar interneurons and sparing of myelin, respectively. RIA values (total and concentration) for GFAP were increased significantly in *jj* subjects, findings consistent with an astrocytic response to bilirubin encephalopathy.

Endogenous phosphorylation of cerebellar proteins. A particulate-bound Purkinje cell-specific phosphoprotein has been characterized recently (Walaas *et al.*, 1985, 1986). Because PCPP-260 represents a major cerebellum-specific protein, it can be identified by protein staining in SDS gels (Walaas *et al.*, 1985, 1986). When proteins from total particulate fractions of cerebellum were resolved by SDS-PAGE, an M_r 260 band was identified by Coomassie blue staining of samples prepared from *Jj* rats (fig. 3); this band was reduced or absent in samples prepared from *jj* rats (fig. 3). No differences were observed between *Jj* and *jj* rats with respect to the staining of other particulate proteins (fig. 3) or the staining of total soluble proteins (data not shown). Because PCPP-260 can be phosphorylated by cAMP-dependent protein kinase, it can also be identified on the basis of its *in vitro* phosphorylation profile. Phosphorylation of particulate fractions from cerebellum by endogenous protein kinases revealed the presence of a major phosphoprotein that comigrated with the M_r 260 stained band (fig. 3). The addition of cAMP to the reaction mixture stimulated the phosphorylation of the M_r 260 band and several other proteins, including two bands with electrophoretic mobilities corresponding to synapsin Ia and Ib (80–85K). Endogenous phosphorylation of the M_r 260 band and several other proteins, both in the presence and absence of cAMP, was reduced in fractions derived from *jj* rats (fig. 3). Overall, these qualitative data indicate that a protein with the electrophoretic and phosphorylation characteristics of the previously described PCPP-260 is reduced in cerebellar particulate fractions from *jj* rats.

Extraction and phosphorylation of PCPP-260. The reduced protein staining and endogenous phosphorylation of PCPP-260 was suggestive of a loss of this protein in *jj* rats. To pursue these initial findings a back-phosphorylation method (Walaas *et al.*, 1985, 1986) was used to quantify PCPP-260 in

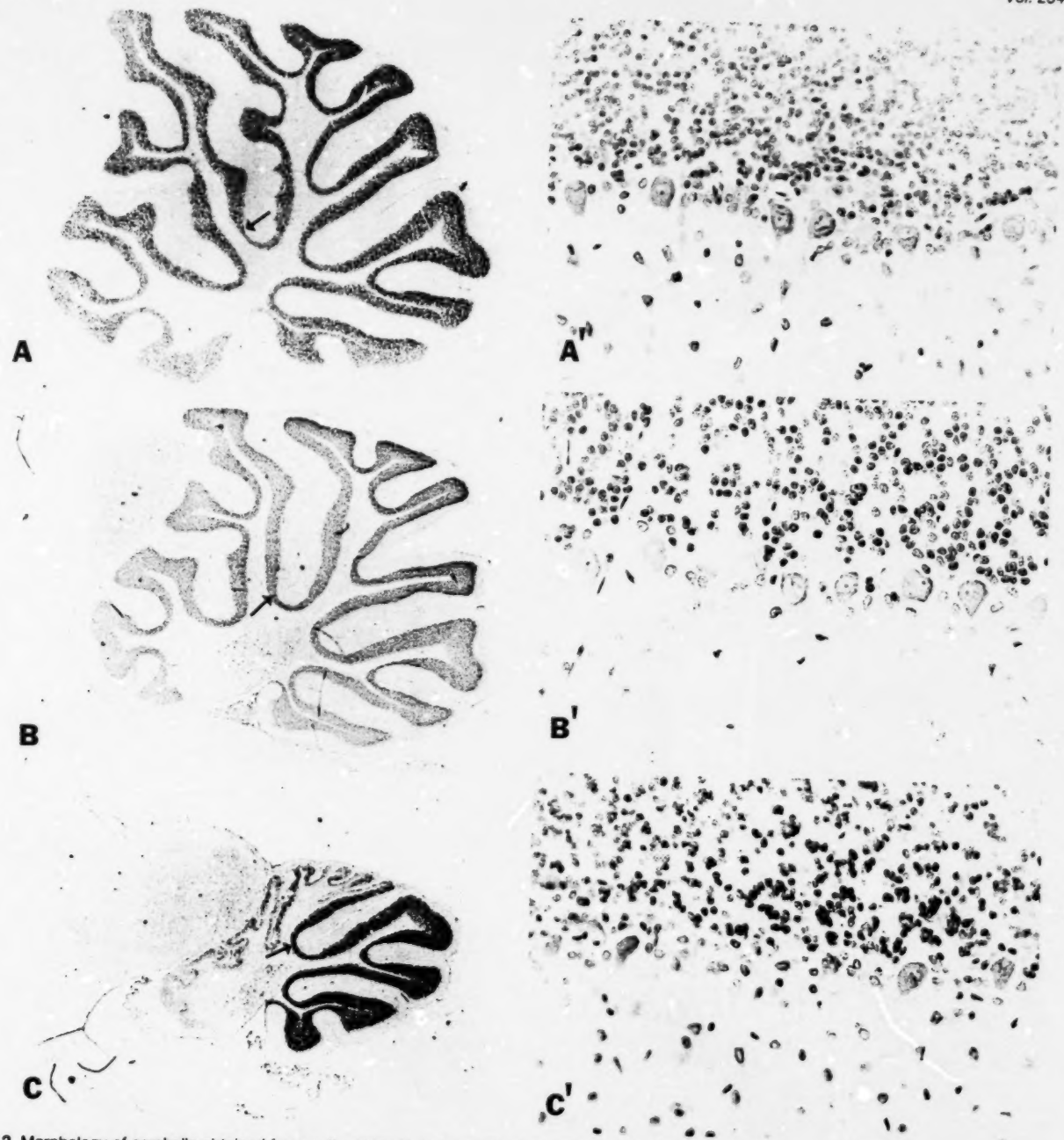

Fig. 2. Morphology of cerebella obtained from a *Jj* control (A, A′), a moderately affected *jj* subject (B, B′) and a severely affected *jj* subject (C, C′). Left panels, saggital sections of *Jj* and *jj* cerebella photographed at the same magnification (×10). Right panels, enlargements obtained from corresponding sections in left panels to show Purkinje cell line (areas enlarged are shown by arrows in left panels) (×250). Note the Purkinje cells in the micrograph from the moderately affected *jj* subject (B′) appear normal whereas those in the micrograph from the severely affected subject (C′) are less numerous, smaller and show the condensed nuclei and hyperchromatosis characteristic of pyknosis.

extracts of cerebellar particulate fractions prepared from *Jj* and *jj* rats. Autoradiographs of phosphorylated cerebellar extracts are shown in figure 3. Comparison of an extract prepared from a *Jj* control with an extract prepared from a severely affected *jj* subject (cerebellar wet weight = 0.090 g) revealed that a phosphoprotein corresponding to PCPP-260 was almost completely absent in the latter sample. In addition, the synapsin Ia, Ib doublet was decreased and an unidentified 55K band was increased in the extract from the *jj* subject (fig. 3). Quantitative data for PCPP-260 are summarized in table 2. In *jj* rats with reductions in cerebellar weight comparable to those obtained in group 2 subjects (see table 1), values for total and concentration of PCPP-260 were decreased to 25 and 40%, respectively, of corresponding values for *Jj* controls. Thus,

TABLE 1
Cerebellar hypoplasia in the Gunn rat is accompanied by changes in neurotypic and gliotypic proteins

	Body Wt.	Serum Bilirubin	Brain Wt.	Cerebellum Wt.	Brain Wt. Minus Cerebellum	RSA*									
						G-substrate		Synapsin I		GFAP		MBP			
						Total	Conc.	Total	Conc.	Total	Conc.	Total	Conc.		
	g	mg/dl	g	g											
Jj[b]	472 ± 4	0.35 ± 0.09	2.12 ± 0.04	0.30 ± 0.01	1.82 ± 0.03	1.00 ± 0.07	1.00 ± 0.09	1.00 ± 0.13	1.00 ± 0.11	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.05		
jj	390 ± 7***	5.27 ± 0.26***	1.88 ± 0.03***	0.15 ± 0.02***	1.73 ± 0.02*	0.17 ± 0.06***	0.36 ± 0.09***	0.33 ± 0.06***	0.63 ± 0.06**	1.66 ± 0.05***	3.81 ± 0.61**	0.60 ± 0.07***	1.23 ± 0.03***		

[a] Each value represents the mean RSA ± S.E.M. expressed on a total (per structure) or concentration (per milligram of protein) basis; see "Material and Methods" for additional details.
[b] Rats were killed at 10 months of age. N = at least 6 for all determinations.
* Significantly different from *Jj* controls, $P < .05$; ** significantly different from *Jj* controls, $P < .01$; *** significantly different from *Jj* controls, $P < .001$.

proteins associated with both the soluble (G-substrate) and particulate (PCPP-260) compartments of Purkinje cells are preferentially decreased as a consequence of bilirubin encephalopathy.

Concentration of neurotypic and gliotypic proteins as a function of cerebellar wet weight. Previous histochemical and biochemical findings have shown that indices of cell loss or damage are inversely correlated with cerebellar wet weight in *jj* rats (Aoki *et al.*, 1982; Keino, 1982). Values for the concentration of neurotypic and gliotypic proteins were plotted as a function of cerebellar wet weight; these data are presented in figure 4. In general, values for concentration of G-substrate, PCPP-260 and synapsin I obtained from *jj* subjects increased in a linear fashion with cerebellar wet weight. In contrast, values for GFAP showed an inverse relationship to cerebellar weight. Decrements in G-substrate and PCPP-260 and increments in GFAP were detected even in *jj* subjects with cerebellar weights that approached those of *Jj* controls. Values for MBP were not correlated with cerebellar wet weight of *jj* rats, and consistent with previous observations (Tamaki and Yamasaki, 1976), the concentration of serum bilirubin also was not related to cerebellar wet weight.

Cerebellar cGMP and cAMP. The concentration of cGMP in hemicerebella from *jj* rats was reduced by 60% relative to values from *Jj* controls (table 3). Three lobules that have different ontogenetic profiles were dissected from the cerebellar vermis of the contralateral hemicerebella. In *Jj* rats the concentration of cGMP did not differ among cerebellar lobules. In contrast, the concentration of cGMP in *jj* rats was reduced in lobule VI but not in lobules V and IX relative to control values. No significant variation in the concentration of cAMP was detected between *Jj* and *jj* samples for any of these analyses.

Neurobehavioral Assessments

Locomotor activity. Deficits in motor behavior have been reported in asymptomatic *jj* rats that correlate with the degree of cerebellar hypoplasia (Tamaki *et al.*, 1977). Within the group of 36 Gunn rats used for the neurobehavioral assessments, the mean cerebellar weight of the *jj* subjects was 55% of the *Jj* controls (0.165 *vs.* 0.298 g; $t = 8.63$, $P < .0001$), a reduction in weight similar to that found for the groups described above (see tables 1 and 2). Assessment of locomotor activity in the figure-8 mazes (fig. 5, left panel) showed the *jj* subjects to be markedly more active in comparison to *Jj* controls with the decline in activity over the test session occurring at the same rate for both groups. When locomotor activity was plotted as a function of cerebellar weight (fig. 6), an inverse relationship was observed between these indices, *i.e.*, the more severe the degree of cerebellar hypoplasia the greater the likelihood that an animal would have a high activity score. One notable exception was the *jj* subject with the smallest cerebellum (fig. 6) whose activity score was not the highest but was still higher than most of the control subjects. Although able to walk, this animal exhibited tremor and was severely ataxic.

Coordination and balance. In the test of coordination and balance, only 75% of the *jj* group was able to successfully negotiate the plank by the end of three trials whereas the *Jj* group experienced little difficulty (95% successful) in mastering this task (fig. 5, right panel insert). Deficits in coordination and balance also were evident in the *jj* animals that were successful in the plank-walk task (fig. 5, right panel). Whereas all successful animals (both *Jj* and *jj*) improved over the three

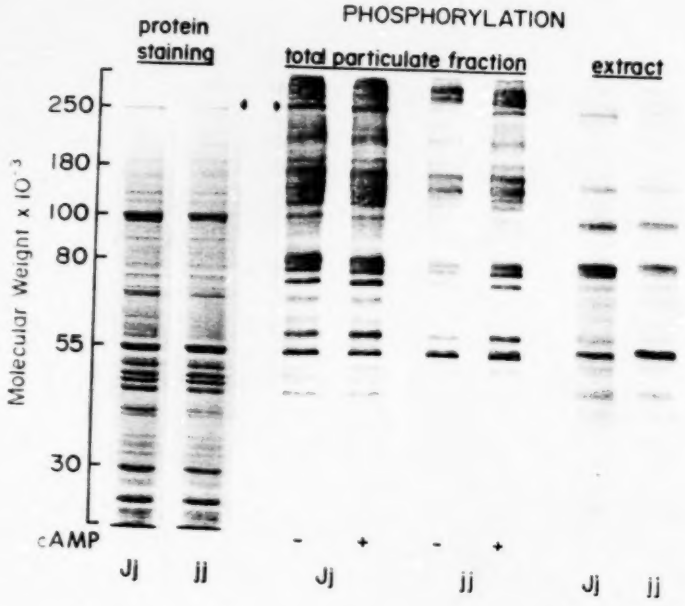

Fig. 3. Protein staining, endogenous phosphorylation and back-phosphorylation of extracts of total particulate fractions prepared from cerebella of *Jj* and *jj* rats. Endogenous phosphorylation reactions were conducted in the absence (−) or presence (+) of 10 μM cAMP. A band of *M*, 260,000 is denoted by arrows. Wet weights of cerebella obtained from the *Jj* and *jj* subjects that were used for protein staining and endogenous phosphorylation were 0.288 and 0.107 g, respectively. Wet weights of cerebella obtained from the *Jj* and *jj* subjects that were used for back-phosphorylation of total particulate fraction extracts were 0.292 and 0.090 g, respectively.

TABLE 2

Cerebellar hypoplasia in the Gunn rat is accompanied by a decrease in PCPP-260

	Body Wt.	Serum Bilirubin	Brain Wt.	Cerebellum Wt.	Brain Wt. Minus Cerebellum	RSA of PCPP-260*	
						Total	Conc.
	g	*mg/dl*		*g*			
Jj[b]	364 ± 12	0.33 ± 0.03	2.00 ± 0.02	0.29 ± 0.02	1.71 ± 0.02	1.00 ± 0.09	1.00 ± 0.03
jj	324 ± 11[c]	7.21 ± 0.37[c]***	1.77 ± 0.06[c]**	0.14 ± 0.03[c]***	1.63 ± 0.03[c]**	0.25 ± 0.10[c]***	0.40 ± 0.10[c]***

* Each value represents the mean RSA ± S.E.M. expressed on a total (per structure) or concentration (per milligram of protein) basis; see "Materials and Methods" for additional details.

[b] Rats were killed at 4 months of age. *N* = at least 6 for all determinations.

[c] Significantly different from *Jj* controls, $P < .05$; ** significantly different from *Jj* controls, $P < .01$; *** significantly different from *Jj* controls, $P < .001$.

test trials, the *jj* rats always required more time to reach the level platform, a finding that was most evident on the initial trial.

Nociception. Sensory as well as motor deficits are associated with the development of hyperbilirubinemia (see Jew and Sandquist, 1979). When tested for responsiveness to a nociceptive stimulus (heat), response latencies recorded for the *jj* group (8.8 sec) were significantly less than those of the *Jj* controls (10.5 sec) ($t = 2.08$, $P < .04$). Thus, *jj* subjects were more sensitive to the nociceptive stimulus in comparison to *Jj* subjects.

Discussion

Hyperbilirubinemia in the Gunn rat is associated with cerebellar hypoplasia and, in severely affected subjects, with permanent alterations in the cytoarchitecture of the cerebellum. Our results demonstrate that these morphological manifestations of bilirubin encephalopathy are accompanied by quantitative changes in proteins associated with specific cerebellar cell types; effects that were accompanied by alterations in neurobehavioral indices of motoric competence and nociception.

All neurotypic proteins examined, G-substrate, PCPP-260 and synapsin I, were reduced in *jj* subjects. When expressed on a per milligram of protein basis (concentration) as well as on a per cerebellum basis (total), the Purkinje cell-specific proteins, G-substrate and PCPP-260, were more affected than synapsin I, a synaptic vesicle localized protein that, in cerebellum, is associated with most interneurons (Dolphin and Greengard, 1981; Dolphin *et al.*, 1983). We interpret these data to mean that Purkinje cells are more affected than cerebellar interneurons in *jj* rats, a conclusion that is consistent with the known cerebellar pathology of the Gunn rat (Schutta and Johnson, 1967; Sawasaki *et al.*, 1976; Mikoshiba *et al.*, 1980; fig. 2, this paper). Because all three neurotypic proteins were reduced in concentration, it is also likely that Purkinje cells and interneurons were decreased to a greater degree than the remaining cerebellar cell types.

The nervous (*nr/nr*) mutant mouse, like the homozygous (*jj*) Gunn rat, exhibits Purkinje cell degeneration. Unlike the Gunn rat, which retains a significant portion of its Purkinje cells for life, nervous mice lose 90% of their Purkinje cells by postnatal day 60 (Landis, 1973). Despite this loss of Purkinje cells, adult nervous mice retain the presynaptic morphology associated with cerebellar interneurons (Sotello and Triller, 1979) and lose only 12% of synapsin I (per milligram of cerebellar protein) (Dolphin and Greengard, 1981). By comparison, we observed a 38% loss in the concentration of synapsin I in cerebella from *jj* rats. Taken together, these data indicate that

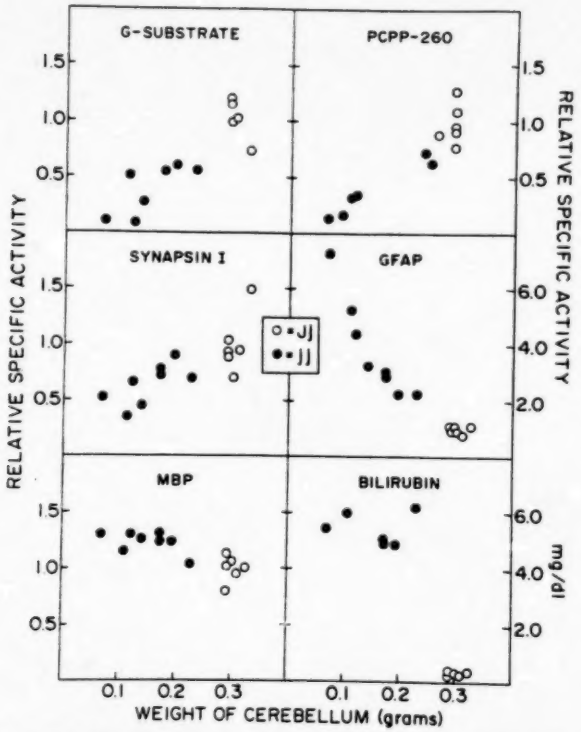

Fig. 4. Relationship between cerebellar weight and concentration of individual neurotypic and gliotypic proteins. All values were obtained from subjects in group 2 with the exception of those for PCPP-260 which were obtained from group 4 (see "Materials and Methods" for additional details). Pearson product moment values for G-substrate, PCPP-260, synapsin I, GFAP, MBP and bilirubin were 0.72, 0.96, 0.68, −0.92, −0.57 and −.07, respectively (*jj* data). Only the PCPP-260 and GFAP values were significant ($P < .001$).

the loss of granule cells or other interneurons in the Gunn rat probably is not secondary to a loss of Purkinje cells but, rather, may be a direct effect of hyperbilirubinemia as suggested previously by Mikoshiba and co-workers (1980).

Immunohistochemical identification of astrocytes using antisera to GFAP indicates that astrocytes undergo hypertrophy in the affected lobes of cerebellar from *jj* rats (Mikoshiba *et al.*, 1980). Our data demonstrate that this astrocytic response is manifested by a marked increase in GFAP. Because toxicant- or trauma-induced injury to the CNS often results in astrocytic proliferation at the site of injury (Latov *et al.*, 1979; Billingsley and Mandel, 1982), increased GFAP probably reflects astrocytosis subsequent to degeneration of Purkinje cells and interneurons. Recently, we observed that toxicant-induced damage to the developing and mature CNS also is associated with a large increase in concentration of GFAP (Brock and O'Callaghan, 1984; J. P. O'Callaghan and D. B. Miller, unpublished observation); however, unlike the condition found in the Gunn rat, this effect was transient with a decline to control values occurring over a period of several weeks. The fact that GFAP remains elevated in cerebella of adult *jj* rats raises the possibility that there is ongoing damage to the cerebellum as a consequence of hyperbilirubinemia.

Because MBP did not decrease as much as cerebellar weight in samples obtained from *jj* subjects, we take this as an indication that myelin-forming oligodendroglia, although perturbed as a consequence of hyperbilirubinemia (*e.g.* see Mikoshiba *et al.*, 1982), were not as affected as other cell types. These findings are in general agreement with the early histological observations of Schutta and Johnson (1967) as well as more recent neurochemical studies (Mikoshiba *et al.*, 1980; Aoki *et al.*, 1982). In these latter studies, the specific activity of CNPase, a putative myelin marker, was either unchanged (Mikoshiba *et al.*, 1980) or slightly increased (Aoki *et al.*, 1982) in samples of cerebellum obtained from *jj* rats. Although Aoki and co-workers (1982) indicate that increased CNPase may be a function of decreased protein concentration in *jj* rats, such an effect could not account for the increase in MBP concentration observed in the present study because cerebellar protein concentration did not differ between *jj* and *Jj* rats (data not shown).

On the basis of protein staining patterns in SDS gels, it has been reported that proteins of M_r 400K (Mikoshiba *et al.*, 1980) and 250K (Aono *et al.*, 1983) are decreased and a protein of M_r 50K (Aono *et al.*, 1983) is increased in cerebella of *jj* rats relative to *Jj* controls. Because the M_r 400K and 250K proteins are apparently identical to PCPP-260 (see Aono *et al.*, 1983; Walaas *et al.*, 1985, 1986), our previous findings, therefore, are consistent with those in the literature. With the exception of the decrease in PCPP-260, we found no consistent differences between *Jj* and *jj* rats with respect to protein staining of particulate or soluble fractions obtained from cerebellum. Phosphorylated extracts obtained from *jj* cerebella, however, did show an increase in a 55K band as well as the expected decreases in PCPP-260 and synapsin I. This 55K band may be identical to the 50K band described by Aono and co-workers (1983). Although we did not attempt to positively identify this protein, we speculate that it may be GFAP, a known phosphoprotein (Browning and Ruina, 1984) of similar M_r (50K) that is increased in cerebella from *jj* rats (table 1; fig. 4).

In previous studies of the Gunn rat the degree of cerebellar hypoplasia has often been linked to the degree of underlying

TABLE 3

Cerebellar hypoplasia in the Gunn rat is accompanied by decreased concentration of cGMP

	cGMP (pmol/mg protein)				cAMP (pmol/mg protein)			
	Hemicerebellum	Lobule			Hemicerebellum	Lobule		
		V	VI	IX		V	VI	IX
Jj[a]	13.93 ± 1.80[b]	14.12 ± 2.25	14.97 ± 2.60	15.32 ± 3.17	15.44 ± 1.57	24.75 ± 5.94	18.88 ± 3.58	18.77 ± 3.73
jj	6.03 ± 1.18***	11.74 ± 1.98	8.93 ± 1.81*	13.78 ± 2.87	15.96 ± 2.05	24.99 ± 3.15	22.66 ± 1.41	20.94 ± 2.08

[a] Animals were killed by focused microwave irradiation. Lobules were dissected according to the method of O'Callaghan *et al.* (1983); see "Materials and Methods" for additional details.

[b] Each value represents the mean ± S.E.M. for at least 17 independent observations.

* Significantly different from *Jj* controls, $P < .05$; *** significantly different from *Jj* controls, $P < .001$.

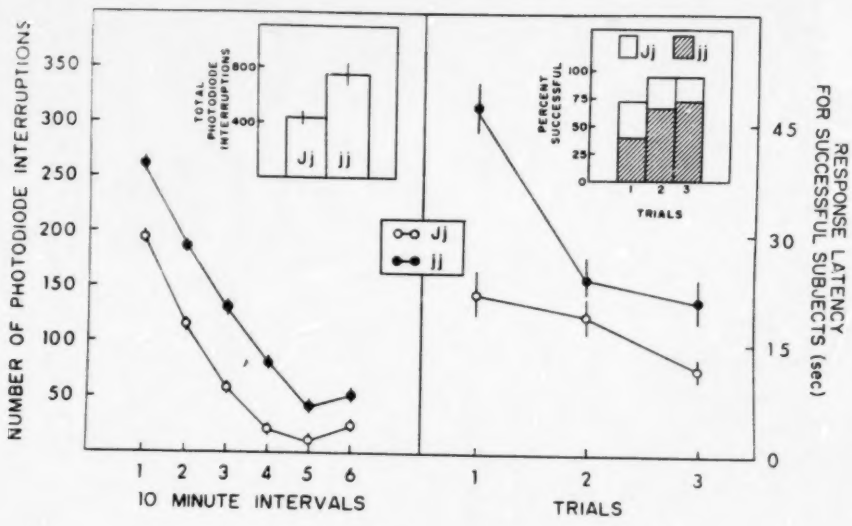

Fig. 5. Motoric competence the *jj* and *Jj* rats as determined by a test of locomotor activity and a test of coordination and balance. Locomotor activity (photocell interruptions) in the figure-8 maze (left panel) expressed as mean interruptions in consecutive 10-min intervals of a 1-hr session and as mean total interruptions (left panel insert). A repeated measure analysis of variance showed an effect of group [$F(1,34) = 22.80$, $P < .0001$] and interval [$F(1,170) = 157.19$, $P < .0001$]. Mean comparisons indicate that *jj* rats were significantly ($P < .05$) more active than *Jj* control rats at all intervals. The right panel insert presents the results from the test of coordination and balance expressed as the percentage of animals in the *jj* and *Jj* groups that were successful at walking down an inclined wood plank and reaching a level platform within 120 sec. More animals were successful in the *Jj* control group ($P < .07$, .05 and .05 for trials, 1, 2 and 3, respectively) using a test for significance of difference between two proportions (Bruning and Kintz, 1968). The right panel also presents the latency in seconds to reach the level platform for the successful subjects in the *jj* and *Jj* groups. The *Jj* subjects reached the platform faster than the *jj* subjects on the first ($P < .01$) and the third ($P < .02$) of the three consecutive trials.

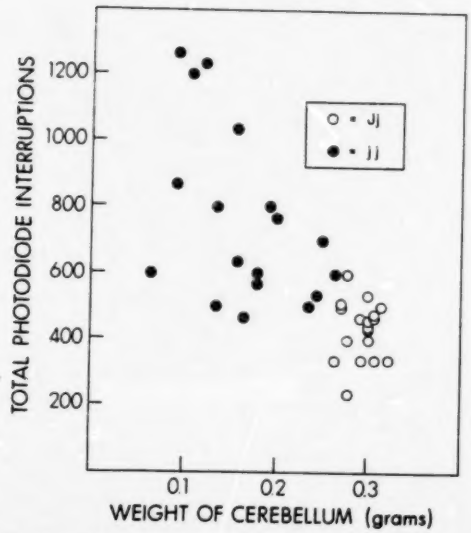

Fig. 6. Relationship between cerebellar weight and locomotor activity. The Pearson product moment value for data obtained from *jj* rats was −0.50 ($P < .036$).

cytopathology as well as the degree of functional impairment (Jew and Sandquist, 1979; Aoki *et al.*, 1982; Keino, 1982). Perhaps it is for this reason that investigations of the neurochemical basis of kernicterus often are conducted with samples obtained from only severely hypoplastic cerebella (*e.g.*, see Mikoshiba *et al.*, 1980). Our data confirm that biochemical indicators of neurotoxicity are most affected in *jj* rats with the smallest cerebella. Thus, the concentration of neurotypic proteins decreased in parallel with cerebellar weight whereas the concentration of GFAP increased in parallel with cerebellar weight. Because the concentration of MBP was not substantially different between *Jj* and *jj* subjects individual RIA values for MBP would not be expected to vary as a function of cerebellar weight. These data, together with the results of the neurobehavioral experiments (see discussion below), indicate that cerebellar wet weight is predictive of neurochemical and functional deficits associated with bilirubin encephalopathy. Because moderately affected *jj* rats do not show overt changes in cerebellar cytoarchitecture (*e.g.*, fig. 2B′) but do exhibit alterations in the amount of specific neurotypic and gliotypic proteins, our data also raise the possibility that assays of these proteins may provide a sensitive method for characterization of the cytopathologic basis of cerebellar neurotoxicity.

Although considerable controversy exists concerning the cellular localization of cGMP in the cerebellum (Cumming *et al.*, 1977; Ferrendelli, 1978; Chan-Palay and Palay, 1979; Ariano *et al.*, 1982), it has been observed in Purkinje cells (Chan-Palay and Palay, 1979). The latter report is consistent with the Purkinje cell specificity of guanylate cyclase (Ariano *et al.*, 1982), cGMP-dependent protein kinase (Lohmann *et al.*, 1978; Schlicter *et al.*, 1980; De Camilli *et al.*, 1984) and its endogenous substrate, G-substrate, (Schlichter *et al.*, 1980; Detre *et al.*, 1984; Nestler and Greengard, 1984). Unlike cGMP, cAMP is evenly distributed in cerebellum and does not appear to be associated with a particular cell type (Rubin and Ferrendelli, 1977). Purkinje cell-deficient mutant mice also show marked reductions in cerebellar cGMP but not cAMP (Mao *et al.*, 1975 and confirmed by us experimentally). On the basis of these previous data it was anticipated that cGMP would be decreased in cerebella of *jj* rats whereas cAMP would not be affected. The data obtained were consistent with our expectations but do not support the hypothesis that cGMP is enriched in or is specific to Purkinje cells. If this were the case, lobule V, which is severely affected in the *jj* rat and is therefore deficient in Purkinje cells (Sawasaki *et al.*, 1976; fig. 2, this paper) should have shown a decrease in cGMP along with lobule VI, a lobule

that also shows a loss of Purkinje cells in *jj* subjects. Thus, it seems probable that cGMP, like cAMP, is not restricted to any one cell type in the cerebellum and consequently would not be suitable as a biochemical indicator of the loss of Purkinje cells.

On the basis of qualitative observations of *jj* rats, we found that overt signs of motoric incompetence (tremor and ataxia) were associated only with subjects presenting severe cerebellar hypoplasia, a finding that is consistent with much of the literature on this mutant (*e.g.*, see Schutta and Johnson, 1967; Tamaki and Yamasaki, 1976). By using objective tests of motor function, one to evaluate general locomotor activity in a novel environment and a more specific test for balance and coordination, we were able to demonstrate that *jj* rats were markedly more active and lacked balance and coordination in comparison to *Jj* controls. Because of the importance of the cerebellum as a neural component in complex motor behavior, it is not surprising that neurotoxic insults that interfere with growth and development of this structure produce permanent alterations in motor function (Lai *et al.*, 1978; Lynch *et al.*, 1976; Phillips and Cragg, 1982; Sager *et al.*, 1984). In agreement with our findings, such alterations in motor function have been shown to include increased exploratory locomotor activity and deficits in fine motor control (Anderson and Altman, 1972).

The strong association between cerebellar size and activity (fig. 6) suggests that cerebellar damage is a major determinant of the changes observed; however, an involvement of other structures cannot be discounted. Alterations in locomotor activity (both increases and decreases) can accompany damage to other brain areas; for example, hippocampus (O'Keefe and Nadel, 1978) and globus pallidus (Norton, 1976). Indeed, damage to brain regions which are thought to primarily process sensory information (*e.g.*, sensory cortex) also can result in decrements in locomotion (Hicks and D'Amato, 1975). In the present study it is possible that other brain areas were damaged because brain weights minus cerebellum were significantly reduced. Although hypoplasia of regions other than cerebellum usually is not reported in *jj* rats, neuronal damage at the ultrastructural level in both cortical and subcortical areas has been observed (Jew and Sandquist, 1979). Thus, despite the importance of the cerebellum in control of coordinated motor movement, damage to other regions of the CNS cannot be eliminated as a factor in the observed functional changes.

Although sensory as well as motor disorders also have been reported in human hyperbilirubinemic populations (see Foley, 1983; Vining *et al.*, 1976 for reviews), sensory deficits have been difficult to demonstrate in the kernicteric Gunn rat (*e.g.*, Butcher *et al.*, 1971; Swenson and Jew, 1982; Tamaki and Yamasaki, 1976). We observed that *jj* rats displayed a peculiar "crossing" of the hind limbs when elevated by the base of the tail; normal rats extend their hind legs when lifted in this manner. This "grasping" reflex has been noted after exposure to toxicants and is purported to be indicative of damage to sensory neurons (see Jolicoeur, 1979). To pursue these initial observations we quantified one aspect of sensory function, responsiveness to a nociceptive stimulus. The data indicated that *jj* rats, rather than showing the expected increased response latencies, responded more quickly than *Jj* controls. It is possible that response latencies in the hot-plate test may be influenced by a number of factors, including those mediated through the peripheral as well as the CNS and that affect motor as well as sensory processes. Therefore, the origin of the hyperreactivity displayed by *jj* rats in this test can not be attributed solely to changes in sensory function. It seems likely that more comprehensive evaluations of sensory function, using tests to evaluate several sensory modalities, will be necessary to characterize sensory status in the Gunn rat.

In conclusion, we have shown that assays of specific neurotypic and gliotypic proteins may be used as biochemical indicators of cerebellar neurotoxicity in the Gunn rat. In comparison to a systematic and comprehensive neuropathological examination, RIAs of these proteins represent a sensitive, more efficient approach to the assessment of cerebellar neurotoxicity. Whether such an approach can be applied to broad classes of neurotoxicants is currently under investigation.

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